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Letter to the Editor

Reoxygenation between Radiotherapy Fractions in an Experimental Osteosarcoma*

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THE TRANSPLANTABLE osteosarcoma C22LR was described earlier [1] as a poorly reoxygenating tumour. Although during fractionated radiotherapy with 3 Gy daily oxygenation of the tumour cells was unaffected, the oxygenation was delayed after large (10 Gy) single doses. At 2 and 3 days after a dose of 10 Gy less than 30% of tumour cells had an oxygen content high enough to ensure normal radiosensitivity [2]. This tumour was extensively studied for its response to hypoxic cell sensitizers [3, 4] and it was concluded that these sensitizers enhanced radiosensitivity only if applied after large doses of radiotherapy, but not after daily fractionated radiotherapy with small dose (3 Gy). Recently attempts were made to study the mechanism of prolonged hypoxia in this tumour. However, no remnants of poor reoxygenation as observed earlier could be found. Since the tumour source, stored in liquid nitrogen, is only four passages away from the tumour studied in 1968, 1976 and 1978, the change in reoxygenation is most probably a consequence of changes in the mouse strains used as hosts for the tumour.

Osteosarcoma C22LR originated in 1957 in a female (C57BL/Rij × CBA/Rij) F_1 hybrid mouse that had been injected with strontium-90. It was serially transplanted in hybrid mice and occasionally stored in liquid nitrogen until a large number of ampoules with cells of passage 75 were frozen in liquid nitrogen in 1967. Around 1975 one ampoule was passaged in mice to obtain large numbers of cells of passage 79, that were again frozen in liquid nitrogen. During this period every 3 months an ampoule was thawed to obtain a fresh sample of these passages for use in experimental studies.

The phenomenon of reoxygenation was studied by determining the fraction of hypoxic cells in the tumour both before irradiation and at 24 and 48 hr after exposure of the tumour to a dose of 10 Gy of X-rays. For determination of the fraction of hypoxic cells groups of mice were exposed to a second dose of irradiation under two conditions: some animals were irradiated alive and other animals were irradiated after asphyxiation with nitrogen. The ratio of the fractions of surviving cells under those two conditions is equal to the fraction of hypoxic cells in the tumour in the living mouse [5].

Cell survival after irradiation was usually studied by the endpoint dilution method [6]: groups of 4–10 tumours were minced with scissors and suspended in Hanks' HEPES balanced salt solution with 0.05% trypsin. After mild agitation for 15 min at room temperature remaining tumour particles were permitted to sediment for 3 min, the suspension was decanted and filtered, and the trypsin was neutralized by cooling and the addition of fetal calf serum. The sedimented tumour fragments were resuspended, the exposure to trypsin was repeated and the cell suspensions obtained were pooled. After counting the cells serial fivefold dilutions were prepared. Aliquots of each dilution were inoculated s.c. at four locations in each of four mice. From periodic observation of these mice the number of tumour takes was determined and this permitted calculation of the cell dose causing 50% tumour takes [5]. In 1984 some of the data on the hypoxic cell fraction in tumours without pre-irradiation were obtained by determining tumour cell survival by clonogenic assay *in vitro*. Since this method did not permit the determination of the low levels of cell survival needed for assessing the fraction of hypoxic cells in pre-irradiated tumours, this technique was used only for tumours that had not been irradiated earlier.

All procedures were as similar as possible to those used for earlier studies [1, 2]. There were, however, minor differences in the method of irra-

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diation. In 1968 mouse irradiation was performed with a Maxitron operated at 250 kV (peak), 30 mA, HVL 2.1 mm Cu at a dose rate of 1.7 Gy/min. In 1984 a Philips–Müller X-ray generator was used at 300 kV (constant potential), 10 mA, HVL 3.2 mm Cu at a dose rate of 1.3 Gy/min. It is assumed that the differences between these irradiation conditions are too small to be responsible for any difference in biological effect.

During attempts to study the mechanism of the poor reoxygenation of osteosarcoma cells after large single doses of radiotherapy, it was noted that reoxygenation occurred normally and rapidly. In contrast to the result of earlier studies, the average hypoxic cell fraction 24 hr after irradiation with a dose of 10 Gy was only 37.2%. In Fig. 1 the data are graphically compared with the results obtained earlier. Notwithstanding the larger variability of the recent results, a statistically significant decrease in hypoxic cell number has occurred ($P < 0.01$).

Analysis of the slope of the survival curve has indicated changes since 1968. Now $D_0 = 3.48$ Gy; in 1968 it was 252 rad. The extrapolation number n now is 1.19; it was 2.30. These changes are associated with a change in the best fit for the fraction of cells surviving after the radiation dose of 10 Gy, that is given to test for the occurrence of reoxygenation. In 1968 this was 2.1%; now it is 6.7%. These changes are just statistically significant at the $P < 0.05$ level.

The comparison of the hypoxic cell fractions as determined at different time points in the history of the osteosarcoma suggests that a real change has occurred in the tumour properties. It appears unlikely that the minor changes in cellular sensitivity to a radiation dose of 10 Gy could be the complete explanation of the apparent good reoxygenation of the tumour. It seems quite likely that a number of independent changes have occurred. The vascularization is a joint function of host and tumour. Since very little change can be expected in

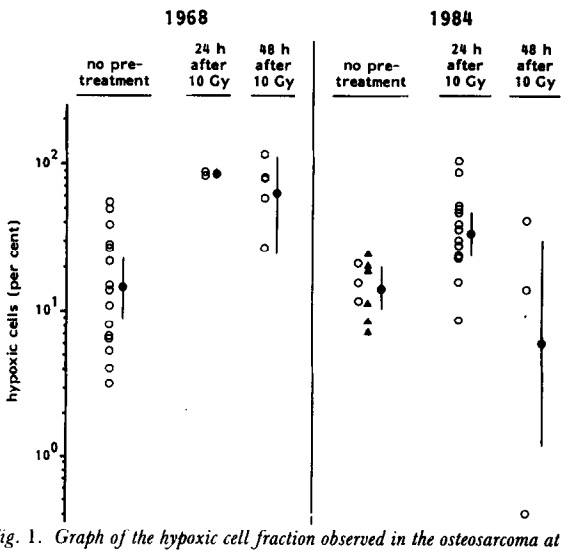

Fig. 1. Graph of the hypoxic cell fraction observed in the osteosarcoma at different times. The open symbols indicate values obtained by serial dilution and transplantation; the closed symbols relate to values obtained by clonogenic assay in vitro. *The error bars marked by the black dots indicate* 95% *confidence limits of the mean. Between* 1968 *and* 1984 *there is a significant* ($P < 0.01$) *decrease in the fraction of hypoxic cells after irradiation.*

the few passages between the samples of tumour maintained in the frozen state, it is much more likely that a change occurred in either the host or the environment. A genetic change may have occurred in one of the inbred parent mouse strains from which the hybrid tumour host mice are produced. However, we cannot exclude that some unidentified factor in the environment or in the food of the mice was responsible for the change.

This tumour was the only one reported in the literature to remain hypoxic for more than 1 day after exposure to a large dose of radiotherapy. No model has been described which remains hypoxic after small doses of fractionated radiotherapy as given clinically. The clinical observations on the failure of hypoxic cell sensitizers to influence the results of fractionated tumour therapy in man [7] are in good agreement with these experimental results.

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